

## PATENT ABSTRACTS OF JAPAN

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### (54) METHOD FOR SUPPRESSING DENATURATION OF PROTEIN

#### (57)Abstract:

PROBLEM TO BE SOLVED: To provide a method for suppressing the denaturation of a protein by heat or an organic solvent to prevent the irreversible formation of aggregates.

SOLUTION: This method for suppressing the denaturation of a protein comprises making FKBP type PPase that is derived from a thermophilic or hyperthermophilic archaea and has a molecular weight from 26 to 33 kDa coexist with the protein.

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**CLAIMS**

[Claim(s)]

[Claim 1] In the amino acid sequence of the protein (b) array number 2 expressed according to the amino acid sequence of the protein (a) array number 2 shown in the following (a) – (c), or four publications, or four publications 1 or two or more amino acid are expressed by deletion and the amino acid sequence permuted or added. According to and the base sequence of the protein (c) array number 1 which has PPIase activity or chaperon Mr. activity, or three publications Protein which DNA with which it is expressed or it and complementary DNA, and DNA hybridized under stringent conditions are protein of the super-thermophilic nature or the thermophile Archea origin which carries out a code, and has PPIase activity or chaperon Mr. activity [claim 2] protein according to claim 1 is coexisted with other protein — making — being concerned — others — the denaturation restraining of the protein characterized by controlling proteinic denaturation.

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## DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Field of the Invention] This invention relates to the approach of controlling the denaturation of the protein produced by environmental stress, such as heat.

[0002]

[Description of the Prior Art] Protein is the polypeptide with which two or more amino acid was connected by peptide linkage. In order for protein to discover the property, the characteristic tertiary structure (spatial configuration) formed of the interaction between intramolecular or a molecule is important. Generally, if protein adds environmental stressors, such as heat, the spatial configuration will change and the property will disappear irreversibly in many cases. Therefore, when how protein is maintained at a stable condition treats protein to such an environmental variation, it is always mentioned as a technical problem. Moreover, in the useful protein production by gene modification technology, purpose protein is produced as an inclusion body with the inactive protein overproduced in host bacteria, such as *Escherichia coli*, and the technical problem that productive efficiency falls is mentioned.

[0003] Research is energetically made as a solution of the above-mentioned trouble until now, and various improvement proposals are proposed. In recent years, the interest is increasing in the molecular chaperone as a factor which participates in proteinic spatial configuration formation and a proteinic structural change. A molecular chaperone is a group of a heat shock protein, and when a cell is exposed to various environmental stress, such as a temperature change, it is produced. These exist widely regardless of a prokaryote and eukaryote, and GroE is well known as a molecular chaperone produced [especially] from *Escherichia coli*. This GroE does not choose a proteinic class but it is shown clearly that it participates in proteinic spatial configuration formation nonspecific. For example, GroEL which is the construct of Above GroE has the characteristic structure that the doughnut mold structure where seven subunits stood in a row annularly lapped with two steps and of consisting of a total of 14 subunits. GroEL catches denatured protein to the crevice of doughnut structure, and folding up to the protein of a right spatial configuration efficiently is known with consumption of nucleotides, such as ATP, and association of GroES which is cofactor. Some attempts which apply this SHAPERONIN to proteinic stabilization are made. For example, "the approach of making an enzyme content solution contain nucleotides, such as SHAPERONIN protein and ATP, and stabilizing the enzyme in a solution" is proposed by JP 7-67641 A. Moreover, "the approach using SHAPERONIN which refined the inactive protein accumulated into the transformant used by the inactive protein which denaturalized chemically, genetic manipulation, etc. from *Thermus thermophilus* (*Thermus thermophilus*) for the purpose of reproducing activity protein" is proposed by JP 7-48398 A.

[0004] When each of these uses a spatial configuration formation operation of the protein which SHAPERONIN has and a spatial configuration deforms them with heat or a modifier, they attain the purpose according to the operation which rewinds a proteinic polypeptide chain to an original spatial configuration (it folds up).

[0005] However, SHAPERONIN needs to make high energy matter, such as ATP, CPT, and UDP, live together generally, in order that [moreover,] SHAPERONIN may act by purpose protein and

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the mole fraction of 1:1 — molecular weight — 1 million — it is necessary to use very high-concentration SHAPERONIN, and economical efficiency is missing in SHAPERONIN of the amount of macromolecules near Da(s).

[0006] On the other hand, PPIase (Peptidyl prolyl cis-trans isomerase) is the cyclosporine and the target molecule of FK506 which are known as an immunosuppressant, and is divided roughly into a cyclo FIRIN type and a FKBP (FK506 binding protein) type from the susceptibility over each immunosuppressant. Moreover, the Per Boleyn (Parvulin) type with which the homologies of an amino acid sequence also differ by the insusceptibility in both immunosuppressant is being found in recent years. Since PPIase had the function (PPIase activity) to promote the reproduction speed of protein higher order structure by carrying out the catalyst of the cis-trans isomerization reaction of the amino terminal side peptide linkage of the proline residue in a polypeptide chain, it was expected as what can be used for proteinic stabilization or playback of inactive protein like above-mentioned SHAPERONIN. Artificers have so far inquired paying attention to the FKBP type PPIase of the Archea origin. Consequently, it has been shown clearly that it has not only the above-mentioned PPIase activity but the activity which increases the yield of the protein volume return originally considered as the function of SHAPERONIN itself and the activity (chaperon Mr. activity) which controls proteinic irreversible condensation in an interesting thing at the FKBP type PPIase of these origins (Maruyama et al., 2000).

FrontBiosci.2000 Sep 1, 5, D 821-836). Unlike the above-mentioned chaperon, PPIase has the advantage that high energy matter called ATP etc. is unnecessary.

[0007] Furthermore, although, as for PPIase marketed until now, Kamiichi of two kinds of a cyclo FIRIN type and FKBP type things [every one kind of] was carried out from for example, the sigma company, respectively, all were the things of the animal origin, and the stability was low and needed to be saved at the low temperature of 4 degrees C or -20 degrees C. Thus, PPIase of the animal origin is very weak with heat. On the other hand, PPIase of the Archea origin does not lose activity, even if it is strong with heat and long duration neglect is carried out under a high temperature service. For this reason, that use is expected as a PPIase reagent with new PPIase of the Archea origin.

[0008]

[Problem(s) to be Solved by the Invention] The great portion of PPIase of the Archea origin is a FKBP type. Moreover, FKBP type PPIase is classified into the thing short type type molecular weight is 17-18 kDa extent, and the thing long type type molecular weight is 26-33 kDa extent (Maruyama, T and Furutani, M Front Biosci.2000 Sep 1, 5, D821-836; Iida et al., 2000, and Gene 256,319-320). The FKBP type PPIase which consists of 26-33 kDa of the Archea origin — the about 17 to 18 kDa from — the field which bears PPIase activity is in the becoming amino terminal domain part, and the remaining C terminal domain part is PPIase of 2 functionality which has the function which controls proteinic condensation, 26-33 Although the amino terminal domain of kDa type PPIase is the high field of a thing short type and homology, compared with a 17-18 kDa type thing, PPIase of the methano KOKKASU thermophilus auto trophy cam (Methanococcus thermoautotrophicum) origin PPIase activity is weak, for example, is [origin] thermophile Archea shows only the activity of or less 1/1000 extent of 16 kDa type PPIase. (deno et al., 2000, Eur.J.Biochem 267, 3139-3148). 25-35 Also in kDa type PPIase, if what shows high efficiency is found out, the utility value is very high. This invention is made under such a technological background, and aims at offering 25-35 kDa type highly efficient PPIase.

[0009]

[Means for Solving the Problem] This invention person completed a header and this invention for having very strong chaperon Mr. activity, although PPIase of pie ROKOKASU HORIKOSHI (Pyrococcus horikoshii) and the METANOKOKKASU YANASHII (Methanococcus jannaschii) origin belongs to a 26-33 kDa type as a result of repeating examination wholeheartedly, in order to solve the above-mentioned technical problem.

[0010] That is, this invention is protein shown in the following (a) - (c).

(a) In the amino acid sequence of the protein (b) array number 2 expressed according to an amino acid sequence the array number 2 or given in four, or four publications 1 or two or more amino acid are expressed by deletion and the amino acid sequence permuted or added.

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According to and the base sequence of the protein (c) array number 1 which has PPIase activity or chaperon Mr. activity, or three publications It is protein of the super-thermophilic nature or the thermophile Archaea origin in which DNA with which it is expressed or it and complementary DNA, and DNA hybridized under stringent conditions carry out a code, the protein which has PPIase activity or chaperon Mr. activity, and this invention coexist the above-mentioned protein with other protein -- making -- being concerned -- others -- it is the denaturation restraining of the protein characterized by controlling proteinic denaturation.

[0011]

[Embodiment of the Invention] Hereafter, this invention is explained to a detail. The protein of this invention contains the protein shown in the following (a) - (c).

(a) In the amino acid sequence of the protein (b) array number 2 expressed according to an amino acid sequence the array number 2 or given in four, or four publications 1 or two or more amino acid are expressed by deletion and the amino acid sequence permuted or added.

According to and the base sequence of the protein (c) array number 1 which has PPIase activity or chaperon Mr. activity, or three publications It is protein of the super-thermophilic nature or the thermophile Archaea origin in which DNA with which it is expressed or it and complementary DNA, and DNA hybridized under stringent conditions carry out a code. Each protein of protein (a) - (c) which has PPIase activity or chaperon Mr. activity is FKBP type PPIase of 26-33kDa. The protein of (a) is PPIase of pie ROKOKASU HORIKOSHI or the METANOKOKASU YANASHI origin.

[0012] The protein of (b) is protein with which the variation of extent which does not make PPIase activity or chaperon Mr. activity lose was introduced into the protein of (a). Such a variation includes an artificial variation besides the variation produced in a nature. As a means to produce artificial variation, although a site-directed-mutagenesis method (Nucleic Acids Res.10, 6487-6500, 1982) etc. can be mentioned, it is not necessarily limited to this. Unless the number of the varied amino acid makes PPIase activity or chaperon Mr. activity lose, although the number is not restricted, usually it is less than 30 amino acid, is less than 20 amino acid

preferably, is less than ten amino acid still more preferably, and is less than five amino acid most preferably. Chymotrypsin couple DOASSEI which Fischer and others proposed can estimate the PPIase activity as used in the field of this invention (Fischer et al., 1984, Biomed.Biochim.Acta 43, 1101-1111). On the other hand, chaperon Mr. activity can use rhodanase, citrate synthetase, a malate dehydrogenase, glucose-6-phosphate dehydrogenase, etc. as a model enzyme (Kawada 1998, bioscience, an industry 56, and 593-598), and can evaluate them by the regeneration rate of denatured protein and the rate of control of condensation of denatured protein which are started when a modifier is diluted with the buffer solution which contains PPIase and SHAPERONIN for these after denaturation processing with protein modifiers, such as 6M guanidine hydrochloride. As an approach Jolo Bitsch's and others approach evaluates (Horowitz, 1995, Methods Mol.Biol.40,361-368), and condensation control of denatured protein, Taguchi's and others approach (Taguchi et al.1994, J.Biol.Chem.269, 8529-8534) etc. is mentioned to the approach of evaluating the regeneration rate of denatured protein, respectively.

[0013] The protein of (c) is PPIase of thermophile Archaea obtained by using the hybridization of DNA, or the super-thermophile Archaea origin. "The stringent conditions" in the protein of (c) means conditions in which only specific hybridization occurs and nonspecific hybridization does not occur. Usually such conditions are "1xSSC, 0.1%SDS, 37-degree-C" extent, are "0.5xSSC, 0.1%SDS, 42-degree-C" extent preferably, and are "0.2xSSC, 0.1%SDS, 65-degree-C" extent still more preferably. DNA obtained by hybridization has DNA with which it is expressed according to the base sequence of array number 1 or array number 3 publication, and usually high homology, high homology -- 60% or more of homology -- desirable -- 75% or more of homology -- 90% or more of homology is pointed out still more preferably.

[0014] The protein of this invention is obtained by the following approaches; for example, First, thermophile Archaea or super-thermophile Archaea is cultivated, and genomic DNA is extracted for the obtained fungus body by technique, such as a phenol extract and ethanol settling, after a bacteriolysis using bacillus solubilizing agents, such as SDS. As the super-thermophilic nature used by this invention, or thermophile Archaea An ASHIDI anus (Acidianus) group, a meta-lass

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FAERA (Metallosphaera) group, A stage OROBASU (Stygiolobus) group, a sulfo ROBASU (Sulfolobus) group, Sulfo ass loess (Sulfolobales) eyes, such as a SURUFUROKOKKASU (Sulfofurococcus) group and a SURUFURISUFAERA (Sulfofurisphaera) group, An AERO pie ram (Aeropyrum) group, a DESURUFUROKOKKASU (Desulfurococcus) group, A SUTETTERIA (Stetteria) group, a SUTAFIRO Thermus (Staphylothermus) group, A thermostat DISUKASU (Thermodiscus) group, an IGUNEOKOKKASU (Igneococcus) group, A SIR MOS FAERA (Thermosphaera) group and a sulfo FOBOKOKKASU (Sulfofobococcus) group, IGUNEOKOKKARESU (Igneococcales) eyes, such as a hyper-Thermus (Hyperthermus) group, a pie RODIKUTITUMU (Pyrodicticum) group, and a pie ROROBASU (Pyrolobus) group, A pie ROBACYURAMU (Pyrobaculum) group, a thermostat Proteus (Thermoproteus) group, Thermostat Protea loess (Thermoproteales) eyes, such as a thermostat FIRAMU (Thermofilum) group and a cardo KOKKASU (Caldococcus) group, AKIOGUROBA loess (Archaeoglobales) eyes, such as an AKIOGUROBUSU (Archaeoglobus) group and a ferro GUROBUSU (Ferroglobus) group, A methano sir mass (Methanothermus) group, the Methanobacterium (Methanobacterium) group, Methano bacteria loess (Methanobacteriales) eyes, such as a methano thermostat BAKUTA (Methanothermobacter) group and a methano SUFAERA (Methanosphaera) group, A methano KOKKASU (Methanococcus) group, a methano thermostat KOKKASU (Methanothermococcus) group, Methano KOKKARESU (Methanococcales) eyes, such as a methano cardo KOKKASU (Methanocaldococcus) group and a meta-noy GUNISU (Methanoginis) group, A methano micro BAIARESU (Methanomicrobiales) eye, Methano ZARUCHINARESU (Methanosarcinales) eyes, such as a methano ZARUCHINA (Methanosarcina) group, Thermostat KOKKARESU (Thermococcales) eyes, such as a methano pillar loess (Methanopyrales) eye, a pie ROKOKKASU (Pyrococcus) group, and a thermostat KOKKASU (Thermococcus) group, Archea, such as thermoplasma loess (Thermoplasmatales) eyes, such as a thermostat plasma (Thermoplasma) group and a PIKURO philus (Picrophilus) group, is mentioned. It is desirable to use the thing of the super-thermophile Archaea origin also in these Archea, and it is desirable to especially use Archea of a pie ROKOKKASU group and Methanococcus.

[0015] Thus, the obtained genomic DNA is connected with a suitable vector after cutting with a suitable restriction enzyme, and a genomic DNA library is produced. The plasmid vector of phagemid DNA, such as lambdaZAP, the various vectors 10, for example, lambdaBAGT, of the lambda phage origin, or a pUC18 and pBR322 grade can be used for a vector.

[0016] On the other hand, DNA which is equivalent to it based on the amino acid sequence of the high field of a homology is compounded between the FKBP type PPIase genes of the different living thing kind origin, and it considers as the primer used for PCR. As an example of the high field of a homology, the 25-32nd amino acid sequences in PPIase of drawing 1, the amino acid sequence of 137 to 144 number, etc. can be mentioned, for example, if PCR which uses the above-mentioned genomic DNA as mold using such a primer is performed, the partial fragment of the gene which carries out the code of the protein of this invention can be obtained. The above-mentioned partial fragment can be used as a probe of a gene screening by carrying out an indicator with radioelements, such as [<sup>32</sup>P], and nonradioactive compounds, such as JIKOKISHI genin.

[0017] What is necessary is to introduce the above-mentioned genomic DNA library into hosts, such as Escherichia coli, and just to choose the clone strongly combined with the above-mentioned probe which carried out labeling, in order to acquire all the base sequences of the gene which carries out the code of the protein of this invention. It can opt for the decision of a base sequence by the Sanger's method or the general approach of the maxam-gilbert method. With the above procedure, all the DNA sequences that carry out the code of the protein of this invention containing a termination codon can be isolated from a translation initiation codon. It is possible to carry out extensive preparation of the protein of this invention by inserting suitably DNA which carries out the code of the isolated protein of this invention in expression vectors, such as a pET system, introducing into a microorganism or a cultured cell and making them discovered by the above-mentioned actuation.

[0018] What is necessary is just to make target protein coexist with the protein of this invention for denaturation control of unstable protein. It not only excels in thermal resistance, but the

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protein of this invention demonstrates depressor effect to proteinic various denaturation in order to show the outstanding cold resistance, organic solvent-proof nature, etc. As a means made to live together, a means to mix the protein of this invention, a means to coexpress the protein of this invention with DNA which carries out the code of the target protein, etc. can be mentioned to target protein, for example. When mixing with target protein, the ratio is a mole ratio and the ratio of 0.1-500 is suitable for it to purpose protein 1. Moreover, coexpression should just insert in the downstream of the manifestation promoter of for example, a pACYC system plasmid the gene which carries out the code of the protein of this invention. For example, when purpose protein is inserted in the expression vector with the DNA replication initiation field of colE1 systems, such as pET, since a pACYC vector can live together within them and a host bacterium, if another drug resistance marker is made to own, both gene lives each plasmid together within a host. The manifestation of both gene is attained under each promoter control. Conversely, even if it builds purpose protein into the pACYC system and builds the PPIase gene in ColE1 system, it does not interfere. If this coexpression system is used, when discovering purpose protein with Escherichia coli etc., for example, it becomes possible to make a solubilization fraction produce the protein discovered as denaturation objects, such as an inclusion body. If the protein of this invention is coexpressed, while the denaturation of purpose protein will be controlled, inclusion body formation is barred by effectiveness, such as PPIase activity and chaperon Mr. activity, and it is produced as a soluble fraction. The protein of this invention is excellent in thermal resistance, and advantageous in the cases, such as conveyance and preservation.

[0019] Hereafter, although an example explains this invention, the range of this invention is not limited to this.

[Example 1] Cloning of the preparation PPIase gene of the genomic DNA of super-thermophile Archea was carried out from pie ROKOKKASU HORIKOSHI and METANOKOKKASU YANASHII. pie ROKOKKASU HORIKOSHI (MD151) came to hand from the Institute of Physical and Chemical Research, and received METANOKOKKASU YANASHII from DSM.

[0020] It is the bacillus suspension of pie ROKOKKASU HORIKOSHI and METANOKOKKASU YANASHII, respectively About 50 ml extraction was carried out and fungus bodies were collected by carrying out at-long-intervals alignment separation for 15000 rmx 5 minutes. After 500micro of TE buffer solutions I washed the fungus body twice, it suspended in TE buffer solution which contains SDS 0.1%, and bacteriolysis processing was performed for 60 minutes at 95 degrees C. After equivalent TE buffer-solution saturation phenol and chloroform performed deproteinization processing, each genomic DNA was settled in ethanol.

[0021] [Example 2] Based on the array information on the pie ROKOKKASU HORIKOSHI origin PPIase shown in magnification, cloning drawing 1, and drawing 2 of a PPIase gene, and the array information on the METANOKOKKASU YANASHII origin PPIase, the PPIase (it is hereafter written as "PHFK") gene of the pie ROKOKKASU HORIKOSHI origin and the PPIase (it is hereafter written as "MJFKL") gene of the METANOKOKKASU YANASHII origin were amplified by the PCR method. As a primer for gene amplification, about PHFK, PHFK-F1 and PHFK-R1 were used, and MJFKL-F1 and MJFKL-R1 were used about MJFKL, respectively (Table 1). The restriction enzyme site was established in these primers. Moreover, a reaction presentation and reaction cycle of PCR are as being shown in the table 2 \*\* table 3, respectively. DNA polymerase used TaKaRaEx.Taq.

[Table 1]  
PPIase 遺伝子及び MJFKL 遺伝子の増幅に用いたプライマー

名称	配列	制限酵素
PHFK-F1	5'-CCATATGTAAGGTGGAGAGGAGATGTT-3'	Nde I
PHFK-R1	5'-GGAAGCTTTTAAAGATGCGCTCTTC-3'	Hind III
MJFKL-F1	5'-CCATATGTAAGAGGAGGTAATGCTA-3'	Nde I
MJFKL-R1	5'-GGGATGCTATTGCTTCTTTCTTTAGT-3'	Bam III

アンダーライン: 各制限酵素サイト

[0023]

[Table 2]  
PCR の反応組成

Reaction buffer(x10)	10μl
dNTP	8μl
Ex. Taq	0.5μl
テンム DNA (10ng/μl)	2μl
Reverse Primer(20pmol/μl)	4μl
Forward Primer(20pmol/μl)	4μl
滅菌水	71.5μl
合計	100μl

[0024]

[Table 3]  
PCR の反応条件

プレヒート	95℃×5min	1cycle
変性	96℃×0.5min	20cycle
アニーリング	59℃×1min	
増幅	72℃×1min	

[0025] The band part which includes after separation each magnification product acquired by the PCR method for a DNA fragment by agarose gel electrophoresis 2% was started, and phenol chloroform processing and ethanol precipitate extracted the purpose DNA. A DNA fragment is dissolved in sterilized water and it is the pT7 blue T plasmid vector of the amount of 10 times to each about ten to 100 ng. (Novagen) In addition, ligation of the DNA fragment was carried out by processing at 16 more degrees C for 1 hour.

[0026] Transformation was carried out by adding the above-mentioned ligation liquid to 109 shares of competent cel Escherichia coli JM, respectively. It is the suspension of these strain 100 mug ml-1 ampicillin sodium and 100 LB agar medium containing muM IPTG and 0.004 % X-Gal was inoculated, it cultivated at 37 degrees C, and about the obtained White colony, PCR which uses the genomic DNA of the strain as mold was performed, and the colony amplified by the primer corresponding to a DNA fragment was made into the electropositive colony overnight. After collecting PTT7 plasmid DNA from an electropositive colony, the base sequence of the acquired PCR product was determined by using a plasmid as mold and performing the sequence reaction (a primer being T7 promoter primer and a U-19 reverse primer) using BIG Dye (PERKIN-ELMER). As a result of comparing this array with the base sequence of the genomic DNA of pie ROKOKKASU HORIKOSHI on a database, or METANOKOKKASU YANASHII, it checked that it was indifferent from the array of a PPIase gene. The amino acid sequence presumed from it by the array number 1 and the array number 3, respectively in the base sequence of the PPIase gene of pie ROKOKKASU HORIKOSHI and METANOKOKKASU YANASHII is shown in the array number 2 and the array number 4, respectively.

[0027] [Example 3] Restriction enzyme processing was performed about each pT7 blue plasmid DNA containing the manifestation system construction PPIase gene of PPIase, and the PPIase gene fragment was cut down. The restriction enzyme used the combination of Nde I and Hind III, respectively. The cut gene fragment is the pET21a plasmid DNA which carried out restriction enzyme processing beforehand by 2% agarose gel after dissociating and extracting. (Novagen) Ligation was carried out. pET21a plasmid DNA is collected from the electropositive colony which contains a PPIase gene after carrying out transformation by adding the obtained ligation reaction mixture to 109 shares of competent cel Escherichia coli JM, and it is competent cel Escherichia coli about this plasmid. BL21 Transformation was carried out to the stock (DE3).

[0028] [example 4] The manifestation of PHFK and MJFKL was tried using the purification profit \*\*\*\* recombination Escherichia coli of PPIase. It is a 2xYT culture medium to the Erlenmeyer flask of 2L. (Yeast Extract 16 g L-1, BACTO TRYPTON 20 g L-1, NaCl5g L-1, ampicillin 100 mug ml-1, pH7.5) 700 ml was put in and two to recombination Escherichia coli 3 platinum loop

containing a PHFK gene or a MJFKL gene was inoculated. After carrying out rotation culture (110 rpm) at 35 degrees C for 24 hours, fungus bodies were collected in centrifugal separation (10000 rpm x 10 min). The obtained fungus body was suspended in 25 mM HEPES buffer-solution (pH 6.8) 20 ml containing 1 mM EDTA, and carried out cryopreservation at -20 degrees C overnight. Column purification was repeated in order of the anion-exchange chromatography of a-d which showed the fungus body to the following after ultrasonic crushing, and gel filtration. When refined fungus body crushing liquid was separated by SDS-PAGE, (the drawing 3 lane 6) was detected as a band also with single case of the Escherichia coli which made MJFKL discover and case of the Escherichia coli which made (the drawing 3 lane 4) and PHFK discover. In addition, the lane 1 in drawing 3 makes a sample the Escherichia coli non-refined fungus body crushing liquid which made PHFK discover, and the non-refined fungus body crushing liquid of the Escherichia coli with which a molecular weight marker and a lane 2 discover the fungus body crushing liquid of wild type Escherichia coli, and the lane 3 made MJFKL discover, and a lane 5 are \*(ng) it.

[0029] a. DEAE Toyopearl column A (16 mm x 60 cm; TOSOH Co., Ltd.) liquid: 25 mM HEPES-KOH The buffer solution (pH 6.8) B liquid: 0.5 M NaCl included 25 mM HEPES-KOH The buffer solution (pH 6.8)-300 min; B liquid 0-100% of straight-line gradient → 300-420 min; The B liquid 100 % rate of flow : [ 1 ml min<sup>-1</sup> ]. HiLoad 26/60 Superdex 200pg column ( [ 26 mm x 60 cm; Amersham ] ) Pharmacia Eluate : 100 mM Sodium phosphate buffer solution ( [ pH 7.0 ; ] 0.15 M NaCl content rate of flow: 3 ml min<sup>-1</sup> ). TSKgel SuperQ-5PW column A (7.5 mm x 7.5 cm; TOSOH Co., Ltd.) liquid: 25 mM HEPES-KOH The buffer solution (pH 6.8) B liquid: 0.5 M NaCl included 25 mM HEPES-KOH The buffer solution (pH 6.8)-10 min; B liquid 0 % → 10-80 min; straight-line gradient rate-of-flow [ B liquid 0-100% of ] → 1 ml min<sup>-1</sup> Id. TSKgel G3000 SWXLcolumn eluate (7.5 mm x 30 cm; TOSOH Co., Ltd.) : 100 mM Sodium phosphate buffer solution (pH 7.0; 0.15 M NaCl content) Rate of flow: 0.5 ml min<sup>-1</sup> [0030] [Example 5] Protein thermal denaturation depressor effect of PHFK (1)

The effect of PHFK to the thermal denaturation of the fungus body crushing liquid digestive liquor of Escherichia coli (BL21 DE3) was considered. That is, ultrasonic crushing of the Escherichia coli which made PHFK discover was carried out, and supernatant liquid was obtained according to centrifugal separation. Protein concentration was carried out in 2.5mg/ml, and it heat-treated for 30 minutes in the temperature requirement of 20 - 100 °C. The protein which remains in supernatant liquid was detected after centrifugal separation by the SDS electrophoresis which used acrylamide gel 16% about supernatant liquid ( drawing 4 ). Moreover, the quantum of the residual protein concentration of supernatant liquid was carried out ( drawing 5 ). As contrast, wild type Escherichia coli was used instead of the Escherichia coli which made PHFK discover, and detection and the quantum of the protein in supernatant liquid were performed.

[0031] As shown in drawing 4 and drawing 5, when PPIase existed, the protein of most Escherichia coli did not condense, but it remained in supernatant liquid. On the other hand, when PPIase did not exist, the amount of detection protein decreased with the rise of processing temperature, and protein hardly remained in supernatant liquid at the time of high temperature processing.

[0032] [Example 6] Protein thermal denaturation depressor effect of PHFK (2) Ultrasonic crushing of the wild type Escherichia coli (BL21 DE3) was carried out, and supernatant liquid was obtained according to centrifugal separation. Protein concentration was carried out in 2.1mg/ml, and further, it added so that it might become the last concentration of 1mg/ml, and PHFK was heat-treated for 30 minutes in the temperature requirement of 20 - 100 °C. The protein which remains in supernatant liquid was detected after centrifugal separation by the SDS electrophoresis which used acrylamide gel 16% about supernatant liquid ( drawing 6 ). The protein which remains in supernatant liquid as contrast, without adding PHFK was detected. [0033] As shown in drawing 6, when PPIase was added, the protein of most Escherichia coli remained, but when PPIase was not added, the amount of the protein detected was decreasing with the rise of processing temperature.

[0034]

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[Effect of the Invention] By this invention, the denaturation of the protein by heat, an organic solvent, etc. can be controlled, and irreversible condensation formation can be prevented. Moreover, the inclusion body formation which poses a problem in recombinant protein production can be controlled according to this denaturation depressor effect. In addition, this invention is [ playback of denatured protein, stabilization of a protein reagent, and a pan ] useful to retrieval of a new immunosuppressant and a physiological active substance.

[0035]

[Layout Table]

SEQUENCE-LISTING <110> MARINE BIOTECHNOLOGY INSTITUTE CO., LTD.<120> METHOD-FOR-RETARDING DENATURATION-OF-PROTEIN<130> P00-0472<160> 8 <170> Patentn Ver. 2.0<210> 1 <211> 774<212> DNA<213> Pyrococcus-horikoshii<220> <221> CDS <222> (1) .. (771) <400> 1 atg aag gtg gag agg gga gat gtt att aag ctc cac tat acc ggt agg 48 Met Lys Val Glu Arg Gly Asp Val Ile Arg Leu His Tyr Thr Gly Arg 1 5 10 15 gtt aaa gag act gga caa ata ttt gac acc act tac gaa gaa gtg gcc 96 Val Lys Glu Thr Gly Gln Ile Phe Asp Thr Tyr Glu Glu Val Ala 2025 30 aaa gaa ggc ata tat aat cca aag ggg atc tac ggtcca gtt cca 144 Lys Glu Ala Gly Ile TyrAsn Pro Lys Gly Ile Tyr Gly Pro Val Pro 35 40 45 ata ato gtc gga gct ggt cac gto att tct gga tta gac aag aag ctg192 Ile Val Gly Ala Gly His Val Ile Ser Gly Leu Asp Lys Arg Leu 50 55 60 gta gga ctt gaa gta gga aag aag tac acc tta gag gtt cca cca gag 240 Val Gly Leu Glu Val Gly Lys Tyr Thr Leu Glu Val Pro Glu 65 70 75 80aaga ttgga cta agg gat ccc aag ctg att aag gta ttc ttt agaaag cag ggg ata gtt cca ttc cca gga tta gaa gta 336 Gly Phe Arg Lys Gln Gly Ile Val Pro Phe Pro Gly Leu Glu Val 100 105 110 gaa gtc acg atc gacaat gga agg aag atg aaa ggt aag gta att aca 384 Gly ValThr Thr Asp Asn Gly Arg Val Met Lys Gly Arg Val Ile Thr 115 120 125 gta agc ggc agt agt gtaga gtt gat ttt aac cac ccc cta gcc gga 432 Val Ser Gly Gly Val Arg Val Asp Phe Asn His Pro Leu Ala Gly 130 135 140 aaa acc ctt attat gag gtagag att gtt gag aag atc gaa gat cca 480 Lys Tyr Glu Val Glu Ile Val Glu Asp Pro145 150 155 160ata gag aag ata aaa gcc cta ata gag ctg agg tta cca atg atc gat 528 Ile Glu Lys Ile Lys Ala Leu Ile Glu Arg Leu Pro-Met-Ile-Asp 165 170 175 agg gat aag gta ata-ato-gaa-gtt-gga gaa aag gat gtt aag gta aca 576 Arg Asp Lys Val Ile Ile-Glu-Val-Glu Lys Asp Val Lys Val-Asn 180 185 190 ttt ggt gacgaat gat gtt gct cccaag acg ctg atc ctg gga gaa att 624 Phe Gly Glu Gln Asp Val Asp Lys Thr Glu Ile Leu Gly Cgc Ile 195 200 205 ctt ttg gag agt gat att aaa ttc ctg gtagat gag aag gtt gaa ttt 672 Leu Leu GluSer Asp Ile Lys Phe Leu Gly Lys Glu Val Phe Cgc 210 215 220 aaa cct agt gtt gaa gag ttgttg agg ccc aag cag gaa gaa ccc gtt 720 Lys Pro Ser Val Glu Glu Leu Arg Pro Lys Gln Glu Pro Val225 230 235 240gaa gag gaa aaa gag gag gag caa gaa agt gaa gag ggc caa toc 768 Glu Glu Lys Lys Glu Glu Gln Glu Glu Ser Glu Glu Ala Gln Ser 245 250 255 tot taa 774Ser(s) <210> 2 <211> 257 <212> PRT <213> Pyrococcus horikoshii <400> 2 Met Lys Val Glu Arg Gly Asp Val Ile Arg Leu His Tyr Thr Gly Arg 1 5 10 15 Val Lys Glu Thr Gly Gln Ile Phe Asp Thr Tyr Glu Glu Val Ala 20 25 30 Lys Glu Ala Gly Ile Tyr Asn Pro Lys Gly Ile Tyr Gly Pro Val Pro 35 40 45 Ile Val Gly Ala Gly His ValIle Ser Gly Leu Asp Lys Arg Leu 50 55 60 Val Gly Leu Glu Val Gly Lys Lys Tyr Thr Leu Glu Val Pro Glu 65 70 75 80 Glu Gly Phe Gly Leu Arg Asp Pro LysLeu Ile Lys Val Phe Thr Met 85 90 95 Gly Gln Phe Arg Lys Gln Gly Ile Val Pro Phe Pro Gly Leu Glu Val 100 105 110 Glu Val Thr Thr AspAsn Gly Arg Met Lys Gly Arg Val Ile Thr 115 120 125 Val Ser Gly Gly Arg Val Arg Val Asp Phe Asn His Pro Leu Ala Gly 130 135 140 Lys Thr Leu Ile Tyr Glu Val Glu Ile Val Glu Lys Ile Glu Asp Pro 145 150 155 160 Ile Ile Glu Lys Ile Lys Ala Leu Ile Glu-Leu-Arg-Leu-Pro-Met-Ile-Asp 165 170 175 Arg Asp Lys Val Ile Glu Val Gly Glu Lys Asp Val Lys-Val-Asn 180 185190 Phe Gly Glu Gln Asp Val Pro Lys Thr Leu Ile Leu Gly Glu Ile 195 200 205 Leu Leu Glu Ser Asp Ile Lys Phe Leu Glu Tyr Glu Val Glu Phe 210 215 220 Lys Pro Ser Val Glu Glu Leu Leu Arg Pro Lys Gln Glu Pro Val 225 230 235 240 Glu Glu Lys Glu Glu Ser Glu Glu Ala Gln Ser 245 250 255Ser <210> 3 <211> 696 <212> DNA <213> Methanococcus jannaschi<220> <221> CDS <222> (1) .. (693) <400> 3 atg gta gaa agt aag atg gta aag att gac tat gac gac tac gtt 48 Met Val Glu Lys Met Val Lys Ile Ser Tyr Asp Gly Tyr Val 1 5 10 15 gat gga aaa cta ttt gat aca act aac gaa gaa ttg gct aaa aag gag 96 Asp Gly Lys Leu Phe Asp Thr Asn Glu Glu Leu Ala Lys Lys Glu 20 25 30 ggg att tac aac cct gca atg attat ggt cct ggt att ttt ggt

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[Translation done.]

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- 1.This document has been translated by computer. So the translation may not reflect the original precisely.
- 2.\*\*\* shows the word which can not be translated.
- 3.In the drawings, any words are not translated.

## DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

- [Drawing 1] Drawing showing the amino acid sequence presumed from the base sequence of a PHFK gene, and it
- [Drawing 2] Drawing showing the amino acid sequence presumed from the base sequence of a MJFKL gene, and it
- [Drawing 3] Electropherogram of the fungus body crushing liquid of the Escherichia coli which discovers MJFKL or PHFK
- [Drawing 4] Electropherogram of the fungus body crushing liquid supernatant liquid under PHFK existence and nonexistence
- [Drawing 5] Drawing showing the amount of protein in the fungus body crushing liquid supernatant liquid under PHFK existence and nonexistence
- [Drawing 6] Electropherogram of the fungus body crushing liquid supernatant liquid at the time of PHFK addition and additive-free

[Translation done.]